RESEARCH NOTE

Characterization of two freshwater silurid catfish using conventional and molecular cytogenetic techniques

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Introduction

Catfish family Siluridae includes nearly 100 extant species. *Ompok* is an important genus of this family that retains four freshwater fish species in India namely: *O. bimaculatus* (Indian butter catfish), widely distributed in India and other countries of Southeast Asia; *O. malabaricus*, found in Western Ghats of Kerala, Goa and Maharashtra; *O. pabda* (pabdah catfish), distribution confined to Indus and Brahmaputra drainages; and *O. pabo*, mainly found in Ganga and Brahmaputra River systems in North Bengal and Assam, respectively. These species differ among themselves mostly in colour pattern, in addition to minor variation in size of anal fin, barbels and shape of caudal fin lobes. *O. bimaculatus* and *O. pabda* are highly priced and preferable fishes in North India, after *Hilsa*, due to flesh quality and taste.

Cytogenetic mapping and molecular organization of ribosomal and other repetitive DNA sequences have provided important aid in characterization of biodiversity and evolution of the ichthyofauna. Classical cytogenetic studies have determined the diploid chromosome number, constitutive heterochromatin (CH) distribution pattern, and location of nucleolar organizer regions (NORs) by silver nitrate and chromomycin A_3 (CMA_3) staining. In higher eukaryotes, the genes coding major 45S ribosomal RNA occur in tandem arrays at one or several specific regions on chromosomes. Each repeat of ribosomal RNA coding gene (rDNA) unit contains a transcriptional unit, i.e. 18S, 5.8S, 28S, two internal transcribed spacers (ITS 1 and 2) to separate them and two external transcribed spacers (5′ ETS and 3′ ETS) surrounds them. Each transcriptional unit is separated by highly variable nontranscribed spacer (NTS) region. The genes coding for 18S, 5.8S and 28S rRNA are highly conserved among various organisms, although the spacer regions are less conserved. Chromosomal segments harbouring these genes are not termed as NOR, which is often associated with nucleolus. Another tandem repeated minor family gene, coding for 5S rRNA, consists of a highly conserved sequence coding for 120-bp long region that is separated by variable NTS. Unlike 45S rDNA, the 5S rDNA is not normally associated with nucleoli formation. Variations in 5S NTS have frequently been characterized in several organisms by insertions/deletions, mini-repeats and pseudogenes are found useful in evolutionary studies and in species/population specific markers development (Pendas *et al*. 1994).

The fluorescence *in situ* hybridization (FISH) using rDNA probe has advantage over silverstaining because it detects rDNA irrespective of their transcriptional activity. Besides, the mass and intensity of resulting fluorescent signal could reflect the gene copy number arrayed at that site. Few conventional cytogenetic studies have been carried out in *O. pabda* and *O. bimaculatus*, but the molecular cytogenetic data are not available. In the present study, we examined the chromosomal localization and sequences of 18S and 5S rDNAs along with the chromosomal distribution of NORs using silver nitrate, chromomycin A_3 (CMA_3) staining and C-banding for use as marker for taxonomic and phylogenetic implications.

Materials and methods

Specimen collection and chromosome analysis

With the help of local fishermen, live specimens of *O. bimaculatus* ($n = 12$) and *O. pabda* ($n = 10$) were collected from river Ganga near Farakka, West Bengal, India. The specimens of both species were at juvenile stage and sex

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was unidentifiable by visual examination. Metaphase chromosome spreads were prepared from kidney and gill cells using conventional hypotonic treatment, fixation (methanol–acetic acid), flame drying technique described by Bertollo *et al*. (1978). NORs staining with silver nitrate ($AgNO_3$) and chromomycin A_3 (CMA_3) were performed as per the protocol described by Howell and Black (1980) and Sola *et al*. (1992), respectively. The technique described by Sumner (1972) was followed for C-banding and slides were stained with propidium iodide (5 μg/mL) as described by Fontana *et al*. (1998). Blood sample was collected from the specimens for DNA isolation. A total of 25 metaphase plates were observed from each specimen of both the species to establish the model diploid chromosome number.

Genomic DNA isolation and PCR amplification

Genomic DNA was extracted from blood using phenol–chloroform–isoamyl alcohol method. The primers (F1: 5′-CGGCTACCACATCCAAG GAAGG-3′ and R1: 5′-CCTT GTTACGACTTTTACTTCCTC-3′) for 18S amplification were designed from *Cyprinus carpio* sequence (NCBI accession no. AF133089, taken from Singh *et al.* 2009). PCR reaction mixture contained 10× *Taq* buffer, 10 mM dNTPs mix, 10 pmol of each primer, 1 U *Taq* DNA polymerase, and 50 ng of genomic DNA in a final reaction volume of 50 μL. The PCR cycling conditions were: initial denaturation at 95°C for 4 min; followed by 32 cycles of denaturation at 94°C for 35 s, primer annealing at 55°C for 35 s and extension at 72°C for 45 s, followed by final extension at 72°C for 10 min. The 5S amplification was carried out separately in 50 μL reaction mixture containing 10× *Taq* buffer, 10 mM dNTPs mix, 10 pmol of each primer (F1: 5′-TACGCCCGATCTCGTCCGAT-3′ and R1: 5′-CAGGCTGGTATGGCCGTAAGC-3′ taken from Moran *et al*. 1996), 1 U *Taq* DNA polymerase, and 50 ng of genomic DNA. The cycling conditions were: initial denaturation at 95°C for 4 min; followed by 34 cycles of denaturation at 95°C for 35 s, primer annealing at 55°C for 35 s, primer extension at 72°C for 45 s; with post cycling extension at 72°C for 10 min.

Sequencing

Amplified 18S rDNA products were purified by PCR purification kit (Qiagen, Hilden, Germany). The 5S rDNA amplicon generated three bands of approximately 200, 400 and 600 bp in length. The 200-bp long fragment was sliced up from the gel, purified by gel extraction kit (Qiagen, New Delhi, India) and was reamplified with the same primers. The 18S and 5S amplicons were sequenced directly using custom service and submitted to GenBank.

Probe labelling

The amplicons of 18S and 5S rDNA were labelled with biotin (Vector Labs, Burlingame, USA) and used as probe.

Probe hybridization

To localize 18S and 5S rDNAs on chromosomes, FISH was performed using protocol described by Winterfeld and Roser (2007) with minor modifications in posthybridization washing at 45°C. For hybridization, two to three days aged chromosome preparations were baked at 90°C for 1 h. Probe detection was carried out with Fluorescein Avidin DCS. The slide was counterstained with DAPI and mounted in Vectashield mounting medium (Vector Labs, Burlingame, USA).

Microscopy

Metaphase spreads were examined under Leica fluorescence microscope (Illinois, USA) with two band filters for simultaneous visualization of DAPI and fluorescein and images were photographed.

Results

The model diploid chromosome number 54 was observed in *O. bimaculatus* with karyotype consisting of 8 pairs of metacentric (m), 13 submetacentric (sm), 3 subtelocentric (st) and 3 pairs telocentric (t) (figure 1a). In *O. pabda*, the model diploid number was 42 and the karyotype composed

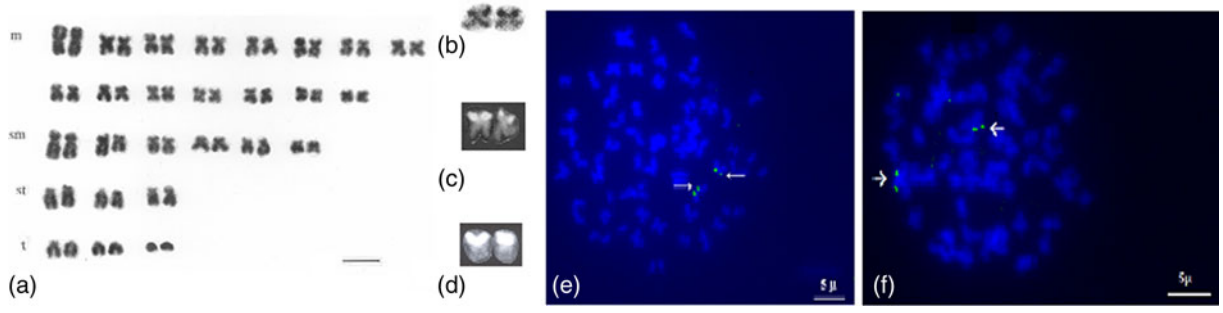

Figure 1. (a) Giemsa stained karyotype; chromosomes showing: (b) silver stained NOR; (c) CMA_3 stained NOR; (d) C-band; metaphase spread showing; (e) 18S; and (f) 5S rDNA-FISH signal (arrow) in *O. bimaculatus*.

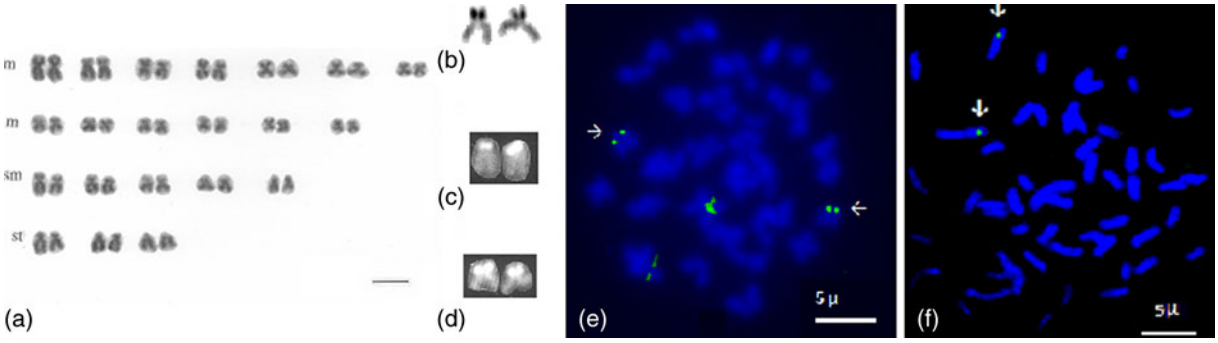

Figure 2. (a) Giemsa stained karyotype; chromosomes showing: (b) silver stained NOR; (c) CMA_3 stained NOR; (d) C-band; metaphase spread showing; (e) 18S and; (f) 5S rDNA-FISH signal (arrow) in *O. pabda*.

13 m, 5 sm, and 3 st chromosomes (figure 2a). The silver and CMA_3 stained NORs and C-banded CH block were present on p arm of one pair of chromosome in both species, but their location in the karyotype was different (figures 1, b–d and 2, b–d). The NOR was present on 2nd m chromosome pair in *O. bimaculatus* and of 1st st pair in *O. pabda*, indicating the presence of species-specific variation in NOR location. The CH block was flanked with NOR. The FISH detected 18S signal, identical to NOR, on one chromosome pair in both species (figures 1e&2e). This indicates the presence of single pair of transcriptionally active NOR. FISH also detected single pair of 5S locus near to centromere of st chromosome in *O. bimaculatus* and sm chromosome in *O. pabda* (figures 1f & 2f).

The 18S rDNA sequencing generated 1811 bp size nucleotide (nt) in *O. pabda* and 1798 bp in *O. bimaculatus* (NCBI accession nos. GU385714 and GU721094). Between the species, 09-nt sequences variable sites, in terms of few base substitutions, deletions or insertions (23 bp) were found. The 18S sequence of *O. pabda* showed 99% identity to the sequence of *O. bimaculatus*. The 5S rDNA size was found to be 202-bp and 201-bp long, in *O. bimaculaus* and *O. pabda*, respectively (accession nos. HM348762 and HM348749). The nt sequence of 5S coding region in subject species was 120-bp long and conserved (100%) which contained three elements, i.e. Box A, Box C and IE, of the internal control regions (figure 3) that functions as a promoter for the gene (Hallenberg *et al*. 1994). The 5S NTS region was found to be 82-bp and 81-bp long in *O. bimaculatus* and *O. pabda*, respectively. The comparative analyses of 5S sequences between the two species showed 36.59% variability in NTS region. The NTS contained TTT, AAATT and TAAT box-like sequences in two species. The coding sequences of 5S in these species showed an average sequence

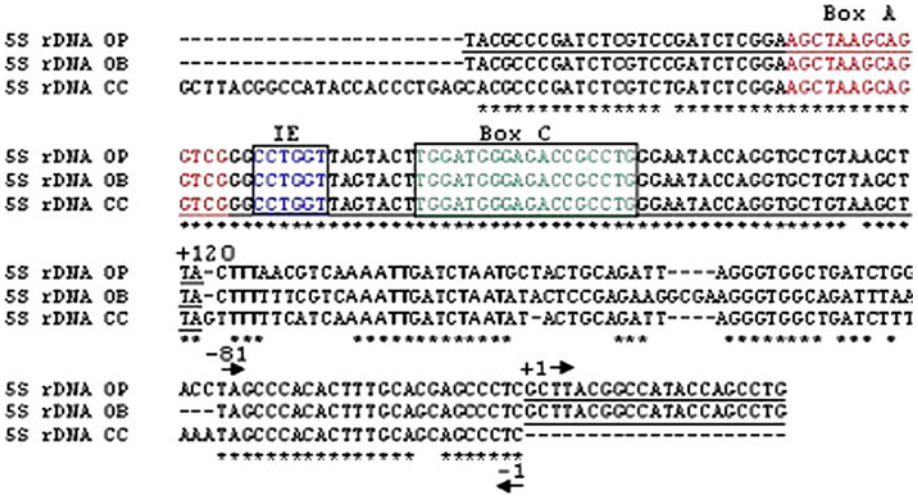

Figure 3. Nucleotide sequences of 5S rDNA in *O. pabda* (5S rDNA OP), *O. bimaculatus* (5S rDNA OB) and *Cyprinus carpio* (5S rDNA CC). The 5S rDNA sequence of *Cyprinus carpio* was taken from NCBI with accession no. AF133089. The coding sequences are underlined. Box A and Box C sequences are indicated in red and green colours, respectively, while IE are indicated in blue colour. TTT like elements are indicated in bold characters.

similarity of >90%, using BLASTN program, with other fishes listed in NCBI.

Discussion

About 12 species, belonging to family Siluridae, have cytogenetically been studied in past (http://www.fishbase.org, version 05/2010). Various workers have reported different diploid number in *O. bimaculatus* ($2n = 40, 41, 42$). Many workers have reported 42 diploid number in *O. bimaculatus* and 54 in *O. pabda* (Nanda 1973; LeGrande 1981; Arkhipchuk Victor V. 1999 Chromosome database. Database of Dr. Victor Arkhipchuk) that may be due to inappropriate identification of the subject species. Further, Arkhipchuk (Arkhipchuk Victor V. 1999 Chromosome database. Database of Dr. Victor Arkhipchuk 1999) and Manna (1989) also reported $2n = 41$ in *O. bimaculatus*. The authors of the present study identified species thoroughly using many keys with large number of specimens. Our finding on *O. pabda* was in agreement with Rahman *et al*. (1995) and Dutta *et al*. (2003). The number and position of 18S and 5S probes were precisely localized on one pair of chromosome in two subject *Ompok* species with positional variation. This is the first report on physical mapping of rDNAs in these species. In these species, the location of silver and CMA_3 stained, and FISH mapped NOR and C-bands could be used in their characterization.

The molecular organization of ribosomal DNA in Indian catfish species is not fully known. The arrangement of 5S rDNA has extensively been studied in animals (Prado *et al*. 1996) and has yielded information on the evolution of 5rRNA gene cluster. In several organisms, the 5S rDNA gene appears strongly constrained to only one chromosome pair, while NORs are often present in multiple chromosomes. Moreover, NOR and 5S rDNA sites may assume a syntenical organization in the same chromosome (Pendas *et al*. 1994; Moran *et al*. 1996) or they can be detected on different chromosomes.

In conclusion, the present study describes the position of major and minor rDNAs and their nucleotide sequences to develop as a species specific marker for two undertaken *Ompok* species. The study showed inter-specific variation in NOR in terms of its position in the karyotype. The nucleotide sequence variation in 18S and 5S rDNAs can serve as a suitable tool for phylogenetic studies. These studies are potentially applicable to other fish species.

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